

### **REMARKS/ARGUMENTS**

Claims 1-10 are currently pending in this application. By the present amendment, claims 3, 5, 6 and 9 have been amended. No new matter has been added. Claims 1, 2, 4, 7, 8 and 10 are canceled herewith without prejudice or disclaimer of subject matter therein or equivalents thereof. The amendment is submitted in response to the Office Action dated March 19, 2009.

#### **I. STATUS OF THE CLAIMS**

Claims 1-2 are rejected under 35 U.S.C. Section 101, (hereinafter, "Section 101, Par. 1") as being directed to non-statutory subject matter as being drawn to a product of nature.

Claims 1-2 are rejected under 35 U.S.C. Section 102(b) (hereinafter, "Section 102(b)") as being anticipated by Haas et al. (GenBank Eccession AY088448, 2002 Genome Biology, hereinafter "Haas").

Claims 1-10 are rejected under 35 U.S.C. Section 103(a) (hereinafter, "Section 103(a)") as being unpatentable over Baucher et al. (1999 Plant Molecular Biology 39:437-447 hereinafter, "Baucher"), in view of Sibout et al. (2003 Plant Physiology 132:848860 hereinafter, "Sibout"), and further, in view of Haas.

#### **Rejection Under Section 101 and Rejection Under Section 102(b)**

Claims 1-2 are rejected under Section 101 and under Section 102(b) as being anticipated by Haas. By the present amendments, claims 1-2 are canceled without prejudice or disclaimer of subject matter therein or equivalents thereof. Accordingly, the rejections of claims 1-2 under Section 101 and 102(b) are believed to be moot.

### **Rejection Under Section 103(a)**

Claims 1-10 are rejected under Section 103(a) as being unpatentable over Baucher, in view of Sibout and further in view of Haas. Applicant respectfully traverses the rejection of the claims and requests reconsideration for all pending claims in light of the remarks below. Claims 3, 5, 6, 7 and 9, have been amended and they are believed to recite patentable subject matter over Baucher, Sibout and Haas as not all the claimed limitations are taught by the cited references.

Applicant respectfully submits that contrary to Examiner's assertions, it is not known that all genes having a function of cinnamyl alcohol dehydrogenase are indispensable for plant growth. As disclosed in T. Goujon et al., Plant Physiology and Biochemistry 41 (2003) 677-687, hereinafter referred to as "Goujon", the CAD-C, D and G genes having a function of cinnamyl alcohol dehydrogenase are shown as not indispensable for plant growth. (See Goujon, Table 4, page 682 and section under subtitle "Cinnamyl alcohol dehydrogenase" on page 684). The Arabidopsis CAD-C, D and D mutants show a normal phenotype in plant growth (see Table 4). In particular, it is described in Goujon that "Enzymatic and chemical analysis carried out on these mutants show that CAD-D is responsible for some lignin features in stems and roots [31]. Further investigations of other single and double mutants of the *CAD* genes are underway to identify the function of each member of this family." (See Goujon under subtitle "Cinnamyl alcohol dehydrogenase" p. 685).

Furthermore, the down-regulation of the *CAD 1* gene of *Nicotiana tabacum* does not lead to inhibition of plant growth as shown in I. Damiani et al., Plant Molecular Biology (2005) 59:753-769, hereinafter referred to as "Damiani". The mutant lines display no phenotypical differences even if the *CAD 1* gene was down-regulated by RNAi. As Damiani describes, "Under all growth conditions (in vitro, growth chamber and greenhouse), *CAD1* down-regulated lines displayed normal growth and morphology. They were also fully fertile." (see Damiani, under subtitle "Plant transformation and phenotypic analysis", p. 760).

Moreover, Baucher, as cited in the Office Action, also mentions that the down-regulated CAD lines of alfalfa do not have any phenotypic differences when compared to the control. (Baucher, first column, p. 441).

Accordingly, such experimental results as shown in the references provided, do not teach or suggest to those skilled in the art nor make obvious that the down regulation of the gene of Applicant's claimed invention causes inhibition of plant growth. Therefore, the amended claims are believed novel and patentable over the cited references. It is respectfully requested that the rejection of the claims under Section 103(a) be withdrawn as the amended claims 3, 5, 6 and 9 are believed to recite novel and patentable subject matter.

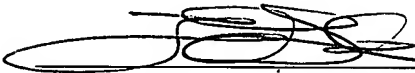
Reference has been made to the Goujan and Damiani documents which have been disclosed in an Information Disclosure Statement herewith filed on August 19, 2009 with full copies, and it is respectfully requested that they be entered and considered by Examiner.

## II. CONCLUSION

It is the Applicant's belief that all of the claims are in condition for allowance and action towards that effect is respectfully requested. If there are any matters which may be resolved or clarified through a telephone interview, the Examiner is requested to contact the undersigned attorney at the number indicated.

Respectfully submitted,

Date: August 19, 2009



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Review

## Genes involved in the biosynthesis of lignin precursors in *Arabidopsis thaliana*

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Received 6 December 2002; accepted 12 February 2003

### Abstract

Lignin is a complex polymer assembled from monolignol precursors derived from phenylalanine after several hydroxylation and methylation steps of the aromatic ring and reduction of the lateral chain. Three main monolignols, the *p*-coumaryl, coniferyl and sinapyl alcohols, give rise, respectively, to the hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units of the polymer. A complete inventory of the genes potentially involved in the monolignol pathway in the model plant, *Arabidopsis thaliana*, is presented in this review. Genes encoding enzymes implicated in constitutive lignin synthesis were identified on the basis of their homology to monolignol biosynthesis genes of other plants and their high expression in lignified tissues (floral stems, roots). This overview shows that most of these genes belong to multigene families and that some (*PAL*, *4CL*, *CAD*) are duplicated in this model plant. The genes encoding the cytochrome P450 monooxygenases (*C4H*, *C3H*, *F5H*) are unique except for *F5H* that has at least one homologue gene present in the complete genome. Mutants and transgenic *Arabidopsis* lines deregulated in the monolignol biosynthesis pathway are listed and the impact of the target gene deregulation on growth, and lignin content and structure are reported.

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**Keywords:** *Arabidopsis*; Lignin; Monolignol biosynthesis; Mutant; Secondary cell wall

### 1. Introduction

Lignin is a plant phenolic biopolymer of complex structure made up of three main *p*-hydroxycinnamyl alcohol precursors or monolignols, namely *p*-coumaryl, coniferyl and sinapyl alcohols. Lignin (from the Latin *lignum*: wood), a characteristic feature of secondary cell walls, accounts for 20–30% of the dry mass of wood, second only to cellulose. Its appearance in evolution is linked to the development of

the upright growth habit of terrestrial plants. Mechanical support and water conductive properties of vascular tissues are augmented by embedding specific cells like vessel elements and fibers with lignin. The lignification of tissues is also part of the defense arsenal of plants to limit pathogen invasion.

The monolignols differ structurally from one another by the number of methoxyl groups present on their aromatic ring; they possess zero, one and two methoxyl groups, respectively (Fig. 1). Lignification is the process of polymerization of the monolignols, *p*-coumaryl, coniferyl and sinapyl alcohols, each giving rise to the hydroxyphenyl (H), guaiacyl (G) or syringyl (S) lignin units, respectively. Deposition of lignin in the cell wall occurs simultaneously as the polymer is formed. Lignin units are linked by different bond-types within the same lignin macromolecule [11,24]. The most frequently encountered linkage is the labile  $\beta$ -O-4 ether bond, which is the target of most degradation techniques used to analyze the chemical structure of lignins and of delignification processes like pulping and bleaching. Lignin

**Abbreviations:** CAD, cinnamyl alcohol dehydrogenase; Cald5H, coniferaldehyde 5-hydroxylase; AldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; CaMV, cauliflower mosaic virus; CCoAMT, caffeoyl coenzyme A *O*-methyltransferase; CCR, cinnamoyl coenzyme A reductase; COMT, caffeic acid *O*-methyltransferase; C3H, coumaroyl coenzyme A 3-hydroxylase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-coenzyme A ligase; EST, expressed sequence tag; F5H, ferulate 5-hydroxylase; G, guaiacyl unit; HCA, hydroxycinnamic acids; PAL, phenylalanine ammonia-lyase; S, syringyl unit.

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Table 4  
Phenotype and lignin characteristics in *Arabidopsis* lines deregulated in the lignin monolignol biosynthesis pathway

Line (gene)	Residual activity	Development (stem size)	Lignin content %/ WT	Lignin composition	Presence of unusual compounds	References
AS4CL ( <i>4CL</i> -1)	8%	Normal	50	Increase S Decrease G		[39]
<i>ref3</i> ( <i>C4H</i> )	ND	Dwarf	Reduced	Decrease S		[53]
<i>ref8</i> ( <i>C3H</i> )	ND	Dwarf	Reduced	H unit		[20,21]
<i>irx4</i> ( <i>CCR</i> -1)	ND	Reduced	50			[29]
ASCCR ( <i>CCR</i> -1)	22%	Reduced	50		Coniferyl and ferulic acids	[22]
<i>fah1</i> ( <i>F5H</i> )	ND	Normal	100	G unit		[10]
35SF5H (Surex <i>F5H</i> )	ND	Normal	100	Increase S		[45,57]
<i>C4HF5H</i> (Surex <i>F5H</i> )	ND	Normal	Reduced	Mainly S	5-OH-G	[42,45]
<i>Atomt</i> -1 ( <i>OMT</i> -1)	15% <sup>1</sup>	Normal	100	G unit	5-OH-G	[23,30]
SOMT-22 (Surex <i>OMT</i> )	285% <sup>1</sup>	Normal	100	WT lignins		[23,30]
<i>Atcad</i> -C ( <i>CAD</i> -C)	77% <sup>2</sup> 34% <sup>3</sup>	Normal	Normal	WT lignin		[58]
<i>Atcad</i> -D ( <i>CAD</i> -D)	20% <sup>2</sup> 2% <sup>3</sup>	Normal	Reduced	Decrease S	Sinapaldehyde	[31,58]
<i>Atcad</i> -G ( <i>CAD</i> -C)	75% <sup>2</sup> 60% <sup>3</sup>	Normal	ND	ND		Eudes et al., unpublished

ND, non-determined; <sup>1</sup>, using stem crude protein extracts and 5-OH coniferaldehyde as substrate; <sup>2</sup> and <sup>3</sup>, using stem crude protein extracts and coniferyl alcohol or sinapyl alcohol respectively as substrates surex, lines overexpressing a specific lignin enzyme.

*At4CL*-3 are less abundant [5]. *At4CL*-1 and -2 are expressed in various tissues but the highest expressions were found in floral stems and roots with a higher level of expression for *At4CL*-1. *At4CL*-3 is expressed at a lower level (only detectable by RT-PCR). Two other *4CL* related sequences (*At4CL*-like 1 and -2) were identified in the *Arabidopsis* genome sequence (Table 1). The only *At4CL*-like ESTs identified in public cDNA databases were three sequences of the *At4CL*-like-2 gene.

No *4CL* *Arabidopsis* mutant has been reported but as mentioned for *PAL* genes, only a double *At4CL*-1 and -2 mutant would be expected to present an obvious phenotype and may be lethal if null. Antisense plants for the *At4CL*-1 gene have been obtained and characterized [39]. Both the *At4CL*-1 and the CaMV 35S promoters have been used with the same efficiency to reduce 4CL activity down to 8% of wild type. When 4CL activity is highly reduced, the *Arabidopsis* transgenic lines possess a normal development in greenhouse with a 50% lignin content reduction and a higher S/G ratio due to an increase in S units and a decrease in G units (Table 4).

### 2.3. Hydroxylation steps (cinnamate-4-hydroxylase, coumaroyl CoA 3-hydroxylase and ferulate-5-hydroxylase)

The first hydroxylation in the phenylpropanoid pathway occurs at the C4 position of the aromatic ring and is carried out by the cinnamate-4-hydroxylase (*C4H*; CYP73A5), a P450-dependant monooxygenase. One gene encoding *C4H* is present in the *Arabidopsis* genome and 16 ESTs are found in cDNA libraries. Northern experiments revealed that *C4H* mRNA is expressed in all tissues with highest levels in stems and roots [5,47]. *C4H* promoter-GUS transcriptional fusion shows a diffuse staining in different tissues with a very high

staining in vascular tissues, such as veins of the leaves, xylem and sclerified parenchyma in the stems [5].

A line named *ref3* for "Reduced Epidermal Fluorescence" was identified [54]; it contains a mutation in the *C4H* gene (cited in [20]). This mutant presents a decreased lignin content and a modified composition (reduction in S units); it also possesses an altered development (dwarf, increased branching, male sterility) [54]. The residual *C4H* activity in this mutant is not reported and may not be null.

The gene involved in the hydroxylation at the C3 position has been identified very recently [55]. The coumaroyl CoA 3-hydroxylase (*C3H*; CYP98A3) is a cytochrome P450 enzyme which catalyzes the 3'-hydroxylation of coumaroyl quinate/shikimate leading to caffeoyl CoA and then to lignin monomers. Present as a unique copy in *Arabidopsis*, it is expressed in all plant tissues (northern experiments and 16 ESTs) but its expression level is by far highest in stems and then in roots and siliques [20,55].

A *C3H* mutant (*ref8*) was obtained and characterized by Franke et al. [20,21]. The mutant is dwarf and possesses a lignin formed primarily from *p*-coumaroyl alcohol, a monomer that is a minor component in the lignin in wild type plants [20]. However, the mutant is probably not null since another *C3H* mutant (T-DNA insertion) was lethal in the homozygous condition (D. Werck, personal communication).

Ferulate 5-hydroxylase (*F5H*) or coniferaldehyde 5-hydroxylase (*Cald5H*; CYP84A1) is a cytochrome P450-enzyme which catalyzes the hydroxylation at the C5 position. This enzyme was first believed to act at the level of ferulic acid but recent studies have demonstrated that *F5H* preferentially hydroxylates coniferaldehyde [27,49] and coniferyl alcohol [27]. The gene was isolated [44] following the

(Table 2). ESTs were found for only 2 of the *AtCCoAOMT* genes (13 for *AtCCoAOMT-1* and 2 for *AtCCoAOMT-2*) in cDNA libraries.

No *Arabidopsis* mutant or antisense lines for *AtCCoAOMT-1* have been reported. However, it is expected that the phenotype of such mutant or antisense lines would be similar to those of antisense tobacco and poplar plants which have a reduced lignin content and a modified lignin composition with a decrease in G units (reviewed in [13,43]).

Caffeic acid *O*-methyltransferase (COMT; EC 2.1.1.68) or 5-hydroxyconiferaldehyde *O*-methyltransferase (AldOMT) was thought to methylate caffeic and 5-hydroxyferulic acids [8]. However, analysis of lignins from COMT antisense plants (reviewed in [4,13,41]) suggested that COMT was preferentially involved in the second methylation step responsible for the formation of S units. More recently, Osakabe et al. [49] and Li et al. [40] demonstrated that COMT preferentially uses 5-hydroxyconiferaldehyde instead of caffeic or 5-hydroxyferulic acids. A *COMT* gene (*AtOMT-1*) was identified by Zhang et al. [69] and its expression determined in some tissues was consistent with a role in lignification. Analysis of the complete *Arabidopsis* genome sequence reveals the presence of at least six other related sequences [23]. Among these genes, *AtOMT-1* is the most closely related to those already reported as involved in lignification in other dicot plants. A role in monolignol biosynthesis is also consistent with its high expression level in stems (Table 3). Numerous ESTs (84) of this gene are present in cDNA databases. This high frequency could also be related to the involvement of *AtOMT1* in both sinapate ester and monolignol biosynthesis [23]. One other member of this class, the *AtOMT-2* gene is also represented by several ESTs [44] but has an unknown role, whereas the other genes are poorly or not represented in cDNA libraries.

The *Atomt1* null mutant [30] possesses a very low *O*-methyltransferase residual activity for caffeic and 5-hydroxyferulic acids, coniferaldehyde and 5-hydroxyconiferyl alcohol [23]. It has a normal development in greenhouse conditions and the same lignin content than the wild type. However, its lignin structure is highly modified since no S units are incorporated in the polymer. S units are partly replaced by 5-hydroxyguaicyl units (5-OH-G) which forms new structures, the benzodioxanes [52]. In addition, the sinapate ester content of this line is reduced [23]. Introduction of a functional poplar *OMT* cDNA is able to complement the *Atomt1* mutant and restores the S/G ratio to the wild type level. However, overexpression of the gene resulting in increased OMT activity had no impact on lignin composition. In contrast, F5H overexpression altered lignin subunit composition significantly [45]. It has thus been concluded that OMT is not the major limiting enzyme for S-unit biosynthesis in xylem of *Arabidopsis* [23,30].

## 2.5. Cinnamoyl coenzyme A reductase

Cinnamoyl coenzyme A reductase (CCR; EC 1.2.1.44) catalyzes the reduction of the hydroxycinnamoyl CoA esters

and is considered as the first committed enzyme of the monolignol specific pathway [36]. Two cDNAs (*AtCCR-1* and *-2*) have been identified and characterized [37]. Expression of *AtCCR-1* was high in stems whereas no expression of *AtCCR-2* was detected in several tissues. In contrast, the level of *AtCCR-2* mRNA increased strongly and transiently after inoculation by a pathogen while the *AtCCR-1* transcript level did not change [37]. These expression patterns are in complete concordance with the number of ESTs found for each gene in cDNA libraries (32 and 6, respectively). Several other related *CCR* genes have been identified by Jones et al. [29], however, their sequences were too divergent to be considered in this work.

The *irx4* (for *irregular xylem*) mutant [63] is mutated in the *AtCCR-1* gene [29]. This line has a dwarf phenotype with collapsed vessels and the lignin content is decreased (50% reduction). An antisense strategy was also used to obtain lines with a 20% residual *CCR* activity [22]. These plants were dwarf and had the same lignin content as *irx4*. In addition, lignins of these antisense *CCR Arabidopsis* are more condensed (less  $\beta$ -O-4 bonds) and contain sinapic and ferulic acids [22] also observed in *CCR* antisense tobacco plants [51].

## 2.6. Cinnamyl alcohol dehydrogenase

Several cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) proteins and genes have been isolated and characterized in different plants (reviewed in [4,13]) but their roles have not been clearly identified. Until recently, CAD proteins were generally believed to catalyze the reduction of the 3 cinnamaldehydes to cinnamyl alcohols, the last step of monolignol biosynthesis. In each plant species, one or two genes were identified as involved in lignification. The isolation of *AtCAD*-related genes in *Arabidopsis* (*Eli3* [34], *CAD-1* [59], *CAD-2* [3]) has been reported with very few indications on their expression pattern. Tavares et al. [62] performed a bioinformatic search for different gene families and identified eight *CAD*-like genes comprising those already known (*Eli3*, *AtCAD-1* and *-2*). Analysis of the complete *Arabidopsis* genome sequence identified one additional gene (*AtCAD-G*; Table 1). A phylogenetic analysis separated the CAD proteins in three main classes [58]. A first cluster includes the two *AtCAD* proteins (*AtCAD-C* and *AtCAD-D*) related to CADs of other plants well documented to be involved in monolignol synthesis. The second cluster is more closely related to the new CAD identified in poplar by Li et al. (named SAD for Sinapyl Alcohol Dehydrogenase [41]) and in alfalfa by Brill et al. (expressed after wounding [7]). *AtCAD-G* constitutes a separate third cluster by itself, with no similar genes cloned in other species. Most of these *Arabidopsis* genes are expressed in stem tissues although different expression levels were observed (Table 2). Only the expression of *AtCAD-C*, *AtCAD-D* and *AtCAD-1* was revealed by northern blot in several tissues, RT-PCR was necessary to detect the others. The number of ESTs in cDNA libraries is in agreement with these results since ESTs of

*AtCAD-1*, *AtCAD-C* and *AtCAD-D* are found most frequently (23, 20 and 25, respectively). Also noteworthy, ESTs of *AtCAD-B1* and *-B2* (also called *Eli 3-1* and *-2*) are relatively frequently encountered (15 and 18, respectively) and are mainly found in plantlet and root cDNA libraries. Their expression has been previously linked to pathogen infection [34]. Until mutants are available, the function of these genes is difficult to verify. In addition, Somssich et al. [60] conducted substrate affinity studies and suggested that *Eli3-2* could be a benzyl alcohol dehydrogenase and not a cinnamyl alcohol dehydrogenase. Recently, we have identified null mutants for *AtCAD-C*, *-D* and *-G* genes ([31,58], Eudes et al., unpublished result). Enzymatic and chemical analysis carried out on these mutants show that *CAD-D* is responsible for some lignin features in stems and roots [31]. Further investigations of other single and double mutants of the *CAD* genes are underway to identify the function of each member of this family.

### 3. Conclusion

This review reports our current knowledge of lignin precursor biosynthesis in *Arabidopsis*. The availability of the complete genome sequence enabled a thorough inventory of putative genes encoding enzymes of this metabolic pathway. Most of the genes belong to small multigene families. Homologies with cDNAs and genes known to be involved in this pathway and published expression studies completed by unpublished data helped to identify which genes may be the most important for the precursor biosynthesis of constitutive lignin. Numbers of ESTs in cDNA libraries are generally consistent with mRNA abundance observed by northern or RT-PCR analysis (as observed for the *AtCAD* gene family). The frequency of ESTs thus constitutes a relatively reliable indicator of gene expression and helps to infer potential involvement in a given pathway. In some cases, expression patterns identified two genes encoding the same enzyme as being involved in this pathway, (*AtPAL-1* and *-2*, *At4CL-1* and *-2*, *AtCAD-C* and *-D*) and in other cases, only one gene seems to be involved (*AtOMT-1*, *AtCCoAOMT-1*, *AtCCR-1*). Chemical analysis of lignin from mutant rachis confirmed the role of some of these genes in lignification. For example, the *AtOMT-1* and the *AtF5H* mutants possess lignins devoid of S units and a *AtCCR-1* mutant contains less lignin. The situation is still unclear for the *AtCAD* gene family where several genes are candidates for a role in constitutive lignification. Moreover, *AtCAD* paralogs may be involved in biotic and abiotic stress, as it was shown for *AtCCR-2*.

The impact of deregulation (under- and over-expression) of specific monolignol biosynthesis genes in *Arabidopsis* is of interest since it seems, in some cases, to be similar to that observed in tobacco plants, in trees like poplar, and in crops like maize and alfalfa. This has been shown for *F5H* [19,45,57], for *CCR* [22,51,63] for *OMT* [4,30] and *CAD* ([31], Lapierre et al., personal communication). In contrast, down-regulation of *4CL* has been shown to induce different

lignin phenotypes according to the target plants (*Arabidopsis* [39], tobacco [32,33], and poplar [26]). Reduction in lignin content was observed in the three species but the impact on lignin structure was different (increase S/G ratio in *Arabidopsis*, decrease S/G in tobacco, no change in poplar). This could be due to the target plant species but also to other factors such as the methods used to analyze lignin (only the non-condensed lignin part is analyzed) and the type of *4CL* genes (*4CL* is a multigene family). A complete collection of single and double *Arabidopsis* mutants with characterized lignin phenotypes could lead to a more comprehensive understanding of the consequences of down-regulation of specific lignin biosynthesis genes and help to develop predictive models for crop plants and trees. Modifications of lignin quality and quantity constitute important targets to improve agro-industrial end uses such as pulp and paper making from forest trees and digestibility forage crop for livestock [6,13,30,43].

### Acknowledgements

The authors are grateful to Nicolas Feau for his helpful assistance with the phylogenetic analysis, to Danièle Werck and Catherine Lapierre for communication of unpublished results.

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## Metabolite profiling reveals a rôle for atypical cinnamyl alcohol dehydrogenase CAD1 in the synthesis of coniferyl alcohol in tobacco xylem<sup>\*</sup>

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Received 23 June 2005; accepted in revised form 14 July 2005

**Key words:** cinnamyl alcohol dehydrogenase, coniferyl alcohol, lignin, xylem

### Abstract

In angiosperms, lignin is built from two main monomers, coniferyl and sinapyl alcohol, which are incorporated respectively as G and S units in the polymer. The last step of their synthesis has so far been considered to be performed by a family of dimeric cinnamyl alcohol dehydrogenases (CAD2). However, previous studies on *Eucalyptus gunnii* xylem showed the presence of an additional, structurally unrelated, monomeric CAD form named CAD1. This form reduces coniferaldehyde to coniferyl alcohol, but is inactive on sinapaldehyde. In this paper, we report the functional characterization of CAD1 in tobacco (*Nicotiana tabacum* L.). Transgenic tobacco plants with reduced *CAD1* expression were obtained through an RNAi strategy. These plants displayed normal growth and development, and detailed biochemical studies were needed to reveal a rôle for CAD1. Lignin analyses showed that *CAD1* down-regulation does not affect Klason lignin content, and has a moderate impact on G unit content of the non-condensed lignin fraction. However, comparative metabolic profiling of the methanol-soluble phenolic fraction from basal xylem revealed significant differences between *CAD1* down-regulated and wild-type plants. Eight compounds were less abundant in *CAD1* down-regulated lines, five of which were identified as dimers or trimers of monolignols, each containing at least one moiety derived from coniferyl alcohol. In addition, 3-*trans*-caffeoyl quinic acid accumulated in the transgenic plants. Together, our results support a significant contribution of CAD1 to the synthesis of coniferyl alcohol *in planta*, along with the previously characterized CAD2 enzymes.

**Abbreviations:** CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; CWR, cell wall residues; F5H, ferulate 5-hydroxylase; GC, gas chromatography; HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; RNAi, RNA interference

<sup>\*</sup> Sequences of *NtCAD1-1* and *NtCAD1-7* were deposited in GenBank under accession numbers AY911854 and AY911855, respectively.

examine the At5g19440 expression pattern. As observed in tobacco, Arabidopsis *CAD1* appears to be expressed in all plant organs and is, in most organs, detected at higher levels than At4g34230 and At3g19450, the two main *CAD2* genes in Arabidopsis (Kim *et al.*, 2004). The Gene Chronologer tool revealed that At5g19440 expression peaks when inflorescence stems develop (21–25 days), but is otherwise steady throughout development.

#### *Plant transformation and phenotypic analysis*

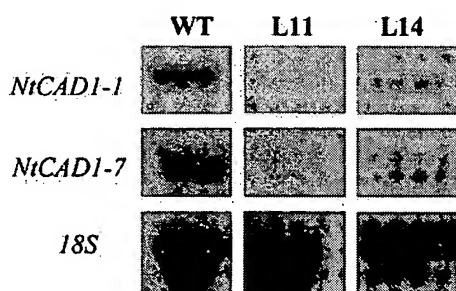
Tobacco was transformed with a construct designed for RNAi-mediated silencing of *CAD1* genes. Although the *NtCAD1-1* sequence was used in the construct, we aimed at silencing both *NtCAD1-1* and *NtCAD1-7* by taking advantage of the high degree of homology between these genes (82% identity at the nucleic acid level in the region used for the RNAi construct). Two independent single copy transgenic lines, L11 and L14, were selected for further analysis. Northern blot experiments showed that the expression of both *NtCAD1* genes was reduced in line L14, and barely detectable in line L11 (Figure 3).

Under all growth conditions (*in vitro*, growth chamber and greenhouse), *CAD1* down-regulated lines displayed normal growth and morphology. They were also fully fertile. Contrary to what was observed for *CAD2* antisense lines (Halpin *et al.*, 1994), no difference in xylem coloration as a result of *CAD1* down-regulation could be noted in any

part of the plant. A microscopic study of transverse stem sections taken from different internodes (examined under white light, for lignin UV-autofluorescence or following staining with phloroglucinol-HCl or Mañile's reagent) showed similar xylem morphology, vessel size and repartition in wild-type and transgenic plants (data not shown). To examine whether *CAD1* down-regulation was compensated for, in transgenics, by an increase in *CAD2* expression, we measured *CAD2* activity in crude xylem extracts using sinapyl alcohol as a substrate. Activities were similar in wild-type, L11 and L14 plants: 582, 580 and 620 nkat/mg protein, respectively. We can thus exclude that *CAD2* enzymes were up-regulated to compensate for *CAD1* silencing.

#### *Analysis of lignin content and composition*

As most monolignols end up in the lignin polymer, we first investigated whether *CAD1* deficiency had any effect on lignin content or composition. Lignin content in the xylem of wild-type and transgenic plants was examined using two different methods. The micro-Klason technique (Whiting *et al.*, 1981) determines the proportion of sulfuric acid-insoluble lignin in cell wall material, thus providing a quantitative estimate of lignin content. Using this technique, similar amounts were obtained for silenced and control lines (Table 2). A qualitative analysis was performed by thioacidolysis according to Lapierre *et al.* (1986). This method allows gas chromatographic detection of lignin monomers released by breakage of  $\beta$ -O-4 intermonomeric bonds. Using this procedure, different results were obtained for the two transgenic lines considered (Table 2). For line L14 in which *CAD1* down-regulation is moderate (Figure 3), we did not observe any differences in non-condensed lignin composition: S, G and S/G values are not statistically different from those measured in wild-type plants. In contrast, for line L11, in which *CAD1* expression is severely decreased (Figure 3), we observed a 32% increase in the S/G ratio. In agreement, the amount of G units relative to Klason lignin content in L11 was significantly lowered by 21% as compared to the wild type. The proportion of monomers involved in  $\beta$ -O-4 linkages was not significantly affected by *CAD1* down-regulation, as shown by the total S + G content relative to Klason lignin (Table 2). This is not



**Figure 3.** Northern blot analysis of *NtCAD1-1* and *NtCAD1-7* expression in wild-type (WT) and transgenic lines (L11 and L14). Twelve micrograms of total RNA extracted from xylem was run on a denaturing gel and blotted onto a nylon membrane. The membrane was hybridized successively with probes corresponding to full-length *NtCAD1-1* and *NtCAD1-7* sequences, and to 18S ribosomal RNA sequence for a quantitation of total RNA loading.